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TECHNICAL MANUSCRIPT 305

AN IMPROVED TISSUE CULTURE MONOLAYER ASSAY FOR VEE VIRUS

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DEPARTMENT OF THE ARMY
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Product Development Division
AGENT DEVELOPMENT AND ENGINEERING LABORATORIES

Project 1B533601D42n

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In conducting the research reported here, the investigators adhered to "Principles of Laboratory Animal Care" as established by the National Society for Medical Research.

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ABSTRACT

A reproducible plaque assay procedure for the quantitation of Venezuelan equine encephalomyelitis (VEE) virus in chick fibroblast monolayer cultures is described that is slightly more sensitive (0.20 log) than the mouse assay. The standard tissue monolayer assay was modified by using tris(hydroxylmethyl)aminomethane (Tris) as a buffer for the agar overlay. Chick fibroblast monolayer cells infected with VEE virus can be maintained under the Tris agar overlay for the time necessary for plaque formation, using a conventional laboratory incubator without a carbon dioxide atmosphere. The adsorption of the virus to chick fibroblast cells was more efficient when the virus inoculum was serially diluted in heart infusion broth at pH 6.0 and when the virus adsorbed for 4 hours at 25 C before the agar overlay was added. Statistically, the plaque and mouse assays showed a linear relationship, 1:1 ratio, between log plaque-forming units and log mouse intracerebral LD_{50} units; thus, the plaque assay can replace the mouse assay for determining virus concentration of VEE products.

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I. INTRODUCTION

Work with Venezuelan equine encephalomyelitis (VEE) virus has been hampered in certain experiments by the lack of an established tissue culture plaque assay procedure that is as sensitive as the mouse intracerebral ($MICLD_{50}$) assay for quantitation of the virus. Quantitative results by the monolayer plaque assay procedure for VEE as described by Hardy and Hearn[1] showed the monolayer plaque assay for VEE virus to be one-tenth as sensitive as the mouse $ICLD_{50}$ assay.

The major objective of this investigation has been to study several environmental variables that could improve the efficiency of the plaque formation, and then to modify the standard procedure by incorporating the improvements to establish a reproducible tissue culture assay procedure equal in sensitivity to the mouse assay for quantitation of VEE virus.

II. MATERIALS AND METHODS

A. VIRUS STRAIN AND WORKING STOCK

The VEE virus, Trinidad strain, was obtained from a donkey with encephalitis and was subsequently passed in embryonated eggs and prepared for use as a stock parent egg seed as described by Hardy.[2]

The working stock virus was either a freeze-dried or a liquid preparation prepared from infected chick embryos, and was stored at dry-ice temperature.

B. MOUSE ASSAYS

Titrations were made by inoculating 0.03 ml of fivefold serial dilutions of the virus in heart infusion broth (HIB) in Swiss-Webster strain mice (10 to 14 g) by the intracerebral route. The mice were observed for 10 days and the deaths recorded. The LD_{50} was calculated by probit analysis.[3]

C. TISSUE CULTURE ASSAYS

Virus concentrations obtained from monolayer tissue cultures are expressed as plaque-forming units (PFU) per ml or g of sample.

D. SOLUTIONS AND MEDIA

Saline A (GKN) and Hanks balanced salt solution (HBSS) were prepared as described by Merchant, Kahn, and Murphy.[4]

1. Trypsin-Versene Solution

This solution is composed of saline A, 1000 ml; trypsin (Difco 1-250), 1.4 g; EDTA (disodium ethylenediamine tetraacetate), 1.4 g. Sterilize the solution by passing through a number 14 Seitz filter. Store the solution at -55 C. Prior to use adjust to pH 8.0 with a 5.6% stock solution of $NaHCO_3$.

2. Growth Media Base[5]

Hanks balanced salt solution, 1000 ml, and lactalbumin hydrolyzate, 5.0 g (Nutritional Biochemicals Corp.) make up this base. Autoclave for 15 minutes at 15 psi and store at 4 C until used.

3. Growth Medium

The growth medium is made from growth media base, 900 ml; calf serum, 100 ml; penicillin G, 2000 units per ml; dihydrostreptomycin sulfate, 2000 micrograms per ml; polymyxin B sulfate, 10 units per ml. Adjust pH to 7.4 with a 5.6% stock solution of $NaHCO_3$.

4. Tris Growth Medium

This medium is identical to the growth medium except the pH is adjusted to 7.4 with a 10% stock solution of Tris (Sigma Chemical Co., St. Louis, Mo.).

5. Nutrient Agar Overlay Medium

The nutrient agar overlay is made from noble agar (Difco), 11 g; gelatin, 5 g; lactalbumin hydrolyzate, 5 g; yeast extract, 1 g; Hanks balanced salt solution without indicator, 1000 ml. Sterilize by autoclaving for 15 minutes at 15 psi and store at 4 C. Prior to use, melt agar overlay in autoclave with free-flowing steam and subsequently transfer to water bath and cool to 44 C. To the melted agar overlay medium add 5000 units of penicillin G per ml, 5000 micrograms of dihydrostreptomycin sulfate, and 30 units of mycostatin per ml. Adjust to pH 7.4 with a 5.6% stock solution of $NaHCO_3$.

6. Tris Nutrient Agar Overlay Medium

This agar is identical to the nutrient agar overlay medium except the pH is adjusted to 7.4 with a 10% stock solution of Tris buffer.

7. Diluents and Adsorption Media

Heart infusion broth (HIB) was prepared in accordance with the formula described by Difco and was used as a standard diluent or adsorption medium for VEE virus. During the experiment, the effect of the adsorption medium pH was studied under different conditions as described in the text.

E. PREPARATION OF CHICK FIBROBLAST MONOLAYER CULTURES

This is based on the procedure of Dulbecco and Vogt[8] with a number of modifications. Sixty 10-day-old embryos were collected in saline A solution. The heads were removed and the embryos rinsed in two changes of saline A. The washed embryos were macerated by pressing them through the orifice of a 50-ml syringe and collected in a flask containing 200 ml of saline A. The tissue pulp was washed in two or three changes of saline A. The tissue pulp was trypsinized with 0.023% trypsin and 0.023% Versene solution prepared by adding 120 ml of trypsin-Versene stock solution to 560 ml of saline A[7] and adjusting the pH to 8.3 with a 5.6% stock solution of $NaHCO_3$. The washed tissue pulp was placed on a magnetic stirrer and trypsinized for 10 minutes at 37 C. The tissue fragments were allowed to settle by gravity and the supernatant fluid was discarded. Trypsinization was repeated by adding 500 ml of trypsin-Versene-saline A solution to the tissue and agitating as before for 25 minutes. The tissue fragments were again allowed to settle and the supernatant cell suspension was dispensed into 250-ml centrifuge bottles. The cells of the supernatant cell suspension were sedimented by centrifuging in a refrigerated International Centrifuge (Model-PR-1) at 650 rpm for 25 minutes. Following centrifugation, the supernatant fluid was discarded and the packed cells were dispensed in known volumes of growth medium; the suspension was filtered through a sterile Rapid-flow filter disk (Johnson and Johnson, Chicago, Ill.) to remove remaining tissue fragments. The cell concentration was determined by counting in a hemocytometer and adjusted by adding growth medium to obtain 30×10^5 to 40×10^5 cells per ml. Ten ml of the adjusted cell suspension was used to seed each plastic (100 x 20 mm) tissue culture petri plate (Falcon Plastics, Div. B-D Labs., Inc., Los Angeles, Calif.). The cultures were incubated 24 hours at 37 C and 85% relative humidity (RH) to obtain satisfactory confluent monolayer cell sheets.

F. INOCULATION OF VIRUS

The growth medium was removed from cultures showing a continuous cell layer and washed with 10 ml of saline A. A volume of 0.2 ml of virus suspension in HIB, at the appropriate dilution, was pipetted onto the cell layer. Adsorption of the virus on the cells was studied by varying the adsorption time and temperatures (see Section III, Results). The infected cultures were covered with 8 ml of melted (44 C) overlay and kept at room temperature for about 10 minutes to allow the agar to solidify. The culture plates were inverted, placed in petri cans, and incubated for 48 hours at 37 C and 65% RH.

C. STAINING OF MONOLAYER FOR PLAQUE COUNTING

After 48 hours' incubation, the plaques on each monolayer were resolved for counting by adding 1 ml of a 1:5000 solution of neutral red to each agar surface and incubating an additional 2 hours to allow for dye penetration.

III. RESULTS

A. COMPARISON OF TRIS AND BICARBONATE BUFFER SYSTEMS

Investigators[8-14] using the plaque technique for titrating some viruses have found that substitution of Tris buffer for bicarbonate buffer in the media gives satisfactory results and eliminates the necessity for a CO_2-containing atmosphere. To evaluate the necessity of a CO_2-containing atmosphere for tissue culture growth and VEE plaque formation, two buffer systems were compared. Parallel sets of chick fibroblast monolayer cultures were prepared. In the standard system, a bicarbonate buffer was used to adjust the growth medium to pH 7.4; in the other system, a Tris buffer was used to adjust to pH 7.4. Petri plates were seeded for both systems with approximately 10 ml of growth medium per plate having a chick fibroblast cell concentration of 30×10^5 to 40×10^5 per ml. The seeded petri dishes for both systems, after incubating for 24 hours at 37 C and 65% RH without atmospheric CO_2, demonstrated confluent monolayer sheets.

The monolayers from both buffer systems were washed with saline A and infected with VEE virus by serially diluting the virus in HIE (pH 7.3) and depositing 0.2 ml of a fixed concentration of virus onto each monolayer. After adsorption for one hour at room temperature, the infected monolayers were overlaid with a nutrient agar overlay medium adjusted to pH 7.4 with either sodium bicarbonate or Tris buffer. The nutrient agar overlay medium was allowed to solidify, the plates were inverted, placed in petri cans, and incubated for 48 hours at 37 C and 65% RH without a CO_2-containing atmosphere.

After 48 hours' incubation the plaques formed on each monolayer were resolved for counting. The data in Table 1 demonstrate that the Tris buffer system can replace the sodium bicarbonate buffer system and eliminate the need for a CO_2-containing atmosphere. Plaque counts obtained with the Tris buffer system compare favorably with those obtained by Hardy et al.[3] that is, the plaque count was approximately one $\log_{10}$ lower than the mouse assay. On the basis of these data all further experiments were carried out with a Tris buffer system without the use of a CO_2-containing atmosphere.

TABLE 1. COMPARISON OF PLAQUE COUNTS OBTAINED WITH A FIXED CONCENTRATION OF VIRUS USING A TRIS- AND A BICARBONATE-BUFFERED SYSTEM

Replicate	No. of Plaques Per Plate[a]	
	Tris Buffer	Bicarbonate Buffer
I	54, 67, 52, 48, 62	0, 0, 0, 0, 0
II	52, 60, 53, 59, 38	0, 0, 0, 0, 0

a. Infected monolayers incubated without a CO_2-containing atmosphere.

B. CONDITIONS FOR EFFICIENT ADSORPTION OF VEE VIRUS ON CHICK FIBROBLAST CELLS

A review of the literature on the adsorption technique used for maximum plaque formation suggests that the adsorption temperature and adsorption period vary with the virus under study. This experiment was designed to study adsorption temperature and adsorption time and to define conditions for optimum adsorption for VEE virus. Adsorption time here means the interval between inoculation of virus on the cells and the application of the nutrient agar overlay medium.

Serial dilutions of VEE virus made in HIB, pH 7.3, were used to infect the washed chick fibroblast monolayers. Variables tested were adsorption temperature (4, 25, and 37 C) and adsorption time (30 and 120 minutes). The infected monolayers were overlaid with a Tris nutrient agar overlay medium after adsorption of the virus for the specified times. Following 48 hours' incubation, the plates were removed and the monolayers were stained with neutral red to resolve plaques for counting. The results shown in Table 2 indicate that adsorption was more efficient at 25 and 37 C than at 4 C, and an increase in plaque formation at all temperatures was noted when the adsorption was extended from 30 to 120 minutes.

TABLE 2. PLAQUE FORMATION FOLLOWING VARIATION OF TIME AND TEMPERATURE

Adsorption Time, min		Number of Plaques per Plate for Indicated Adsorption Temperature and Replicate					
		4 C		25 C		37 C	
		I	II	I	II	I	II
30		11	3	35	6	35	14
		18	7	48	13	34	24
		6	5	26	20	40	18
	Mean	12	5	36	13	36	18
120		18	6	47	19	45	25
		22	5	51	21	48	21
		20	5	39	23	45	28
	Mean	20	5	45.6	21	46	24.6

C. PLAQUE COUNT AS AFFECTED BY pH OF THE ADSORPTION MEDIUM

This experiment was designed to study the effect of pH of the adsorption medium. HIB (adsorption medium) was prepared and divided into three lots. The pH of the first lot was adjusted to 6.0 by adding 1 N HCl. The second lot was adjusted to pH 8.0 by adding 1 N NaOH. The initial pH of the third lot was 7.3 and was not adjusted further. Serial dilutions of VEE virus from the three lots of HIB (pH 6.0, 7.3, and 8.0) were used immediately to infect the washed chick fibroblast monolayers. After adsorption for 120 minutes at room temperature (25 C) the plates were overlaid with Tris nutrient agar overlay medium and the agar was allowed to solidify. Following 48 hours' incubation the plates were removed and the monolayers stained with neutral red to solve the plaque for counting. This experiment, Table 3, demonstrated that plaque formation was significantly affected by the pH of the virus diluent. Titers obtained by serially diluting the virus in HIB adjusted to pH 6.0 showed an increase in plaque titers of 0.61 log compared with the virus obtained with standard HIB, pH 7.3. In contrast, virus titers obtained by diluting in HIB at pH 8.0 yielded lower titers (0.29 log).

TABLE 3. PLAQUE COUNT AS AFFECTED BY pH OF ADSORPTION MEDIUM

Replicate	pH of Diluent	Virus Dilution	Number of Plaques per Plate[a/]	10^9 PFU per ml
I	6.0	$10^{-7.0}$	123, 131, 114, 132, 127, 110, 133, 129, 117, 113, 120, 125, 124, 103	6.0
II	6.0	$10^{-7.6}$	10, 17, 32, 43, 38, 30, 26, 30, 39, 23, 35	4.4
III	6.0	$10^{-7.6}$	20, 21, 20, 49, 34, 30, 23, 44, 37	4.7
I	7.3	$10^{-7.0}$	9, 24, 32, 36, 37, 41, 16, 30, 38, 53, 28, 48, 26, 30, 39	1.6
II	7.3	$10^{-7.6}$	4, 9, 3, 4, 6, 7, 7, 9, 9, 8, 7, 0	0.93
III	7.3	$10^{-7.6}$	8, 0, 8, 3, 6, 14, 18, 22, 0, 11	1.2
I	8.0	$10^{-7.0}$	34, 30, 49, 40, 44, 39, 23, 22, 20, 31, 25, 23, 32, 24, 28	1.5
II	8.0	$10^{-7.6}$	2, 3, 2, 3, 0, 2, 2, 0, 2, 2, 2	0.28
III	8.0	$10^{-7.6}$	4, 1, 6, 14, 8, 6, 3, 4, 3, 4	0.83

a. Plates were inoculated with 0.2 ml of appropriate virus dilution at indicated pH with an adsorption time of 120 minutes at 25 C.

D. STUDIES ON THE EFFECT OF ENVIRONMENTAL VARIABLES FOR PLAQUE FORMATION

Data from the experiments discussed demonstrate that plaque formation depends upon the pH of the adsorption medium, adsorption time, and the adsorption temperature. This experiment was designed to select optimum environmental conditions for plaque formation. Environmental variables tested were the adsorption medium (HIB) at pH of 6.0, 7.3, and 8.0, adsorption temperatures of 25 and 37 C, and adsorption times of 5, 30, 60, 120, 180, 240, and 300 minutes. To test pH variables, washed chick fibroblast monolayer plates were divided into three groups. Serial dilutions of VEE virus from a single pool of stock virus in HIB at pH levels of 6.0, 7.3, and 8.0 were used immediately to infect the washed monolayers. The three groups of infected plates were further subdivided into two groups to determine the effect of adsorption temperature. One group was incubated at 37 C, the other remained at room temperature. To determine the effect of adsorption time at different adsorption temperatures and varying pH of the adsorption medium, the infected monolayers were overlaid with Tris nutrient agar overlay medium at designated time intervals. Following 48 hours' incubation the plates were removed and the monolayers stained with neutral red to resolve the plaques for counting. The results of this experiment are shown in Tables 4A and 4B, and Figure 1. A significant increase in plaque formation was obtained when the virus inoculum was serially diluted in HIB at pH 6.0 and the Tris nutrient agar overlay medium was applied after an adsorption period of 4 hours at 25 C or 5 hours at 37 C. Experiments in which the virus inoculum was serially diluted in HIB at pH 7.3 or 8.0 and the virus was allowed to adsorb for 5 hours at 25 or 37 C gave lower counts. The standard procedure of serially diluting the virus in HIB at pH 7.3 and allowing an adsorption period of 1 hour at 25 C (room temperature) was compared with a modified procedure involving dilution of the virus inoculum in HIB at pH 6.0 and an adsorption period of 4 hours at 25 C. The data in Figure 1 show that approximately 1.05 logs more virus were detected by the modified procedure.

TABLE 4A. INFLUENCE OF pH OF VIRUS DILUENT AND ADSORPTION TIME AT 25 C ON PLAQUE COUNT OF VEE VIRUS

Adsorption Time, min	Rep	No. of Plaques per Plate at Indicated pH and Replicate[a]								
		pH 6.0			pH 7.3			pH 8.0		
		I	II	III	I	II	III	I	II	III
5		3	3	15	4	0	5	2	1	18
		11	5	25	14	0	2	1	0	10
		9	1	15	5	0	5	5	0	12
	Mean	7.6	3.0	18.3	7.6	0	4.0	2.6	0.3	13.3
30		14	1	26	8	0	-[b]	11	0	6
		19	1	23	15	0	5	6	0	4
		19	0	-[b]	4	0	7	5	0	3
	Mean	17.3	0.6	24.5	9	0	6	7.3	0	4.3
60		20	10	16	6	0	5	3	0	4
		14	19	21	15	0	6	6	0	5
		25	14	22	19	0	8	5	0	5
	Mean	19.6	14.3	19.6	10.0	0	6.3	4.6	0	4.6
120		17	3	23	23	0	9	9	1	11
		26	3	29	5	0	5	14	0	6
		26	5	50	14	0	5	13	0	1
	Mean	23.0	4.6	34.0	14.0	0	6.3	12.0	0.3	6.0
180		37	15	-[b]	7	0	-[b]	8	1	17
		38	14	50	23	0	0	8	0	8
		55	11	-[b]	0	0	8	10	0	9
	Mean	43.3	13.3	50.0	10.0	0	4	8.6	0.3	11.3
240		88	40	64	24	0	11	22	0	16
		99	16	57	13	0	22	18	4	19
		84	45	70	24	0	24	20	2	13
	Mean	90.3	33.6	63.6	20.3	0	22.3	20	2.0	16.0
300		60	55	73	16	0	11	25	2	9
		81	50	80	15	2	19	14	5	6
		62	54	62	17	0	17	8	4	9
	Mean	67.6	53.0	41.6	16.0	0.6	15.6	15.6	3.6	8.0

a. All monolayers inoculated with 0.2 ml of $10^{-7.8}$ virus dilution.
b. Bacterial contamination on monolayers.

TABLE 4B. INFLUENCE OF pH OF VIRUS DILUENT, AND ADSORPTION TIME AT 37 C ON PLAQUE COUNT OF VEE VIRUS

Adsorption Time, min		No. of Plaques per Plate at Indicated pH and Replicate[a]								
		pH 6.0			pH 7.3			pH 8.0		
		I	II	III	I	II	III	I	II	III
5		7	3	15	16	0	-[b]	4	2	6
		8	0	27	12	0	-[b]	2	0	11
		13	4	20	10	0	15	3	0	8
	Mean	9.3	2.3	20.6	12.6	0	15	3.0	0.6	8.3
30		14	2	30	13	0	-[b]	5	0	2
		8	11	21	16	0	11	4	0	3
		9	3	19	11	0	5	6	0	11
	Mean	10.3	2.0	23.3	13.3	0	8.0	5.0	0	5.3
60		24	6	42	13	0	4	6	2	7
		22	19	23[b]	12	0	-[b]	4	5	4
		22	9	28	16	0	3	7	0	7
	Mean	22.6	11.3	31.0	13.6	0	3.5	5.6	2.3	6.0
120		27	2	13[b]	19	0	8	22	2	5
		28	2	37	13	0	7	10	1	4
		27	15	47	12	0	13	17	0	-[b]
	Mean	27.3	6.3	32.3	14.6	0	9.3	16.3	1.0	4.5
130		67	16	39	10	0	9	18	1	12
		51	14	31	9	0	10	7	0	0
		46	11	37	25	0	11	7	0	8
	Mean	54.6	13.6	35.6	14.6	0	10.0	10.6	0.3	6.6
240		56	13	50	9	0	16	11	2	3
		82	16	30	11	0	15	18	4	9
		86	21	48	15	0	18	10	2	7
	Mean	74.6	16.6	42.6	11.6	0	16.3	13.0	2.6	6.3
300		95	36	70	17	1	15	13	4	17
		45	58	59	13	0	14	11	0	31
		105	46	60	13	1	22	20	2	10
	Mean	81.6	46.6	63.0	14.3	0.6	17	14.6	3.0	19.3

a. All monolayers inoculated with 0.2 ml of $10^{-7.6}$ virus dilution.
b. Bacterial contamination on monolayers.

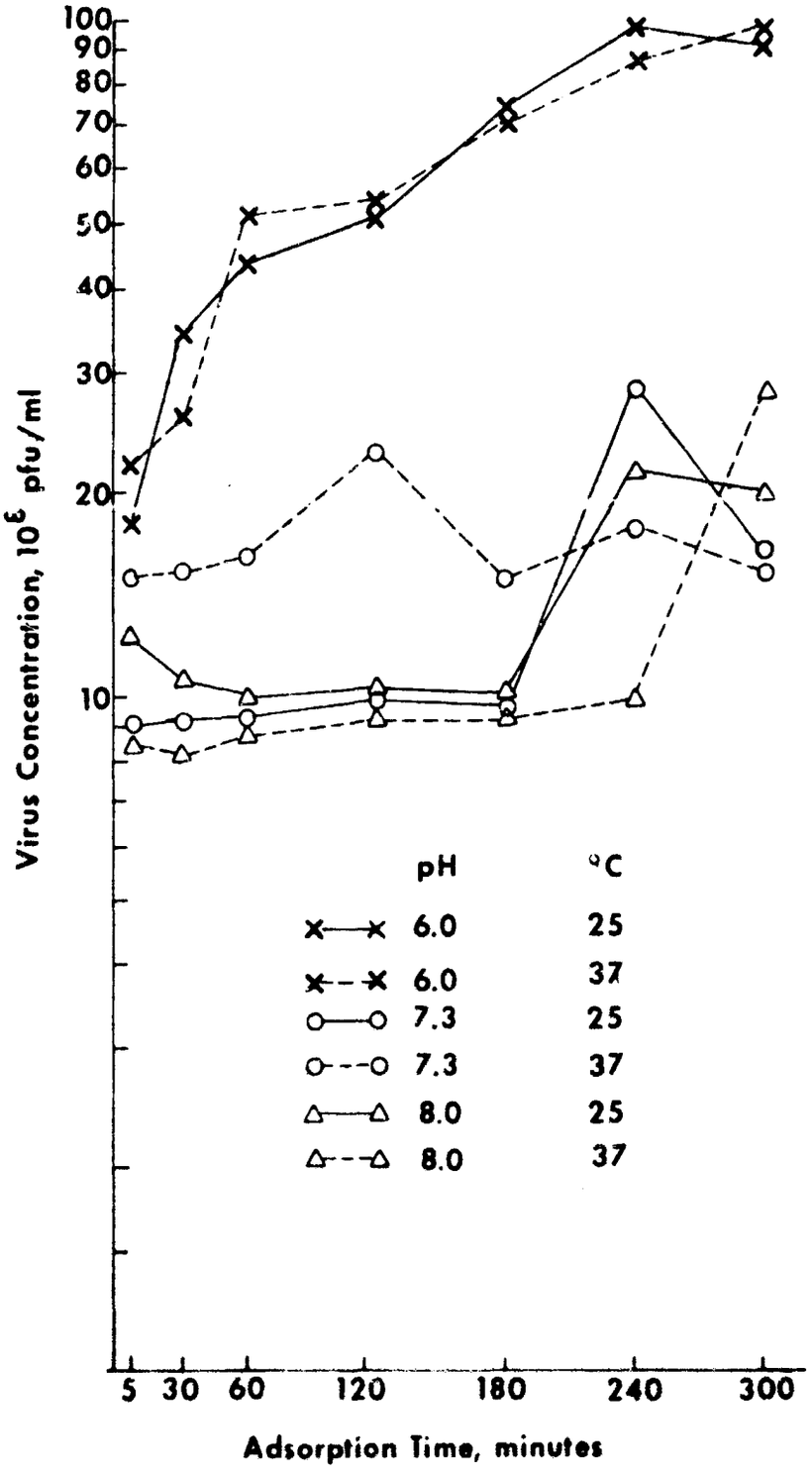

Figure 1. Influence of pH of Virus Diluent and Adsorption Time at 25 and 37 C on Plaque Count of VEE Virus.

E. STUDIES ON THE EFFECT OF PRETREATMENT OF MONOLAYERS AT VARIOUS pH

Finding that the greatest infectivity was obtained when the virus was diluted in HIB at pH 6.0 was unexpected. The mechanism by which an increased plaque count is obtained by pretreating the virus in HIB at pH 6.0 is not known. This experiment was designed to determine if an increase in plaque formation was brought about by pretreatment of the virus particle or the cell of the monolayer at pH 6.0.

Confluent chick fibroblast monolayer plates were divided into two groups. The monolayers from one group were washed with saline A at the usual pH of 7.3; those from the other group were washed with saline A adjusted to pH 6.0. The virus was pretreated by serially diluting in HIB at pH 6.0, 7.3, and 8.0. The monolayer cell sheets for the two groups were infected with appropriate dilutions of virus prepared in HIB with various pH. After adsorption for 4 hours at 25 C, the plates were overlaid with Tris nutrient agar overlay medium and incubated for 48 hours. Then the monolayers were stained with neutral red to resolve the plaque for counting. The results are shown in Table 5. The number of plaques was not increased by pretreatment of the monolayer cell sheet with saline A, pH 6.0. The large increase of plaque formation invariably occurred when the VEE virus was pretreated by serially diluting in HIB at pH 6.0 prior to adsorption on the cells. Thus, one is led to think that the effect is primarily on the virus particle.

TABLE 5. EFFECT OF PRETREATMENT OF MONOLAYERS BY VARIOUS pH

Pretreatment[a] pH	Dilution pH	Rep.	No. of Plaques[b] Per Plate			Mean
6.0	6.0	I	43	49	43	47.1
		II	52	35	61	
7.3		I	36	42	50	49.6
		II	64	51	55	
6.0	7.3	I	0	0	0	11.1
		II	11	21	35	
7.3		I	0	0	0	7.8
		II	12	18	17	
6.0	8.0	I	4	4	8	11.8
		II	16	20	19	
7.3		I	3	10	6	9.6
		II	11	18	10	

a. Chick fibroblast monolayer cells washed with saline A-LX at the designated pH.

b. All plaque counts obtained from a fixed virus concentration treated under different conditions.

F. PLAQUE ASSAY VERSUS MOUSE ASSAY

Two separate but related investigations were conducted to compare the relative merits of the plaque and mouse assay methods. In both investigations, samples of VEE products assayed in tissue culture were serially diluted in HIB (pH adjusted to 6.0) in 0.5-log increments of 8.0, 8.5, 9.0, and 9.5. Five plates with confluent cell sheets of chick fibroblast monolayers were inoculated with each dilution (0.2 ml per plate). An adsorption period of 4 hours was allowed prior to applying the Tris nutrient agar overlay medium. After the agar had solidified, the plates were inverted and incubated for 48 hours at 37 C, RH 65%. Following incubation, the monolayers were stained with neutral red to resolve the plaque for counting. Mouse assays were made by serially diluting the virus sample in HIB (pH 7.3) in 0.5-log intervals of 9.0, 9.5, 10.0, and 10.5. Eight mice were inoculated intracerebrally with 0.03 ml of each dilution. The mice were held in an animal room for 10 days and observed daily for response. The entire procedure was replicated once.

In the first investigation, the virus concentration of three VEE products (A, B, C) was determined by both methods. The number of plaques per plate, the mean plaque count for each dilution, and the variance of the plaque count for each dilution are presented in Table 6. A plot of the variances as a function of the mean, Figure 2, indicates that the variance of the plaque assay procedure is slightly larger than Poisson, and that other sources of variation such as dilution, technician, sheet thickness, etc. exist, but have only a slight effect in this particular situation. The straight line on the graph indicates true Poisson variance.

When determining the virus concentration, dilutions should be prepared so that the number of plaques per plate falls within the linear portion of the curve relating numbers of plaques per plate to concentration. Overcrowding and overlapping of plaques occur, as illustrated in Figure 3; at the higher virus concentrations, these lead not only to inaccurate counts but to excessively large variances (Table 6). At very low concentrations the probability of obtaining a sample devoid of virus particles is greatly increased so that it is difficult to distinguish between a true and a false zero count. In this investigation, counts of approximately 20 to 100 plaques per plate gave the most satisfactory results. The mean number of plaques per plate at each dilution was plotted against dilution of virus on log-log paper as shown in Figure 4. One would expect the slope of this relation to be -1.0 as shown by the straight line, i.e., one would expect the count at the 10^{10} dilution to be one log less than the count at the 10^{9} dilution. Figure 4 shows that the mean plaque counts fall nearly on this line, although there is a suggestion of a slight deviation from linearity at the $10^{9.5}$ dilution.

TABLE 6. NUMBER OF PLAQUES PER PLATE AND THE MEAN AND VARIANCE FOR EACH DILUTION

Rep	Dried Product	Log Dilution	Plates 1	2	3	4	5	Mean	Variance
I	A	8.5	80	138[a]	102	102	135[a]	111.4	605.16
		9.0	45	42	37	52	41	43.4	30.25
		9.5	13	19	23	10	19	16.8	27.04
		10.0	10	5	6	10	7	7.6	5.29
	B	8.5	89	93	96	98	111	97.4	68.89
		9.0	28	26	24	43	32	30.6	56.25
		9.5	9	9	10	10	8	9.2	0.64
		10.0	0	0	1	2	6	1.8	5.76
	C	8.5	186[a]	176[a]	170[a]	116[a]	152[a]	160.0	756.25
		9.0	80	63	79	65	84	74.2	90.25
		9.5	21	21	19	24	26	22.2	7.29
		10.0	7	8	5	9	6	7.0	2.25
II	A	8.5	183[a]	170[a]	150[a]	162[a]	192[a]	171.4	275.56
		9.0	61	70	66	47	67	62.2	81.60
		9.5	29	27	29	25	16	25.2	29.16
		10.0	7	3	5	2	13	6.0	18.49
	B	8.5	103[a]	86	84	102[a]	89	92.8	81.00
		9.0	24	27	29	10	33	24.6	75.69
		9.5	6	6	9	6	7	6.8	1.69
		10.0	6	4	2	1	3	3.2	3.61
	C	8.5	173[a]	193[a]	209[a]	194[a]	175[a]	188.8	201.64
		9.0	65	98	78	68	75	76.8	166.41
		9.5	16	25	26	28	18	22.6	27.04
		10.0	4	7	4	3	3	4.2	2.56

a. Overlapping plaques, very difficult to count accurately. However, date are included in mean values.

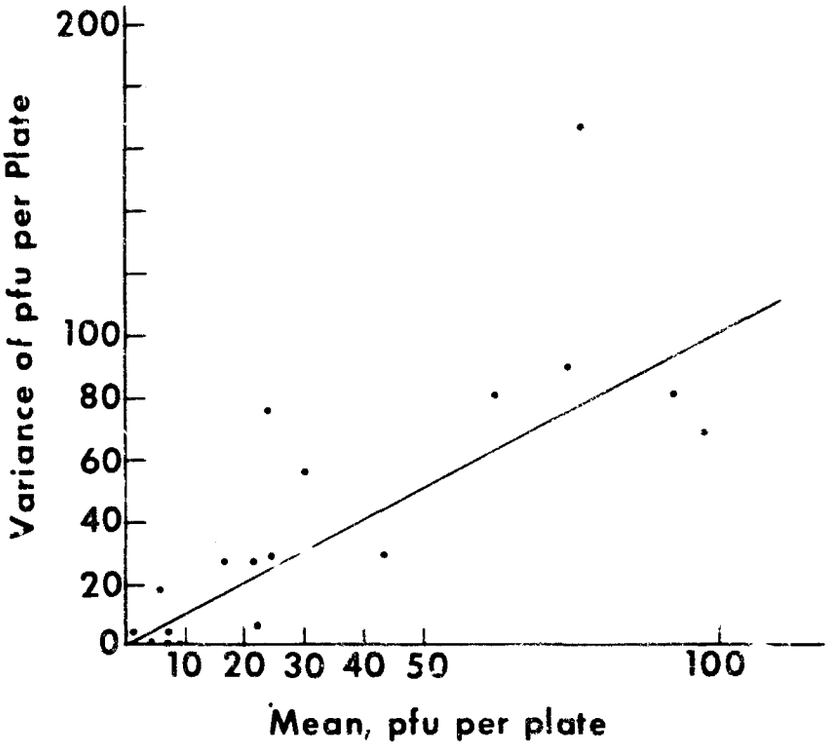

Figure 2. Mean Plaque Count (0 to 100) vs. Variance of Count.

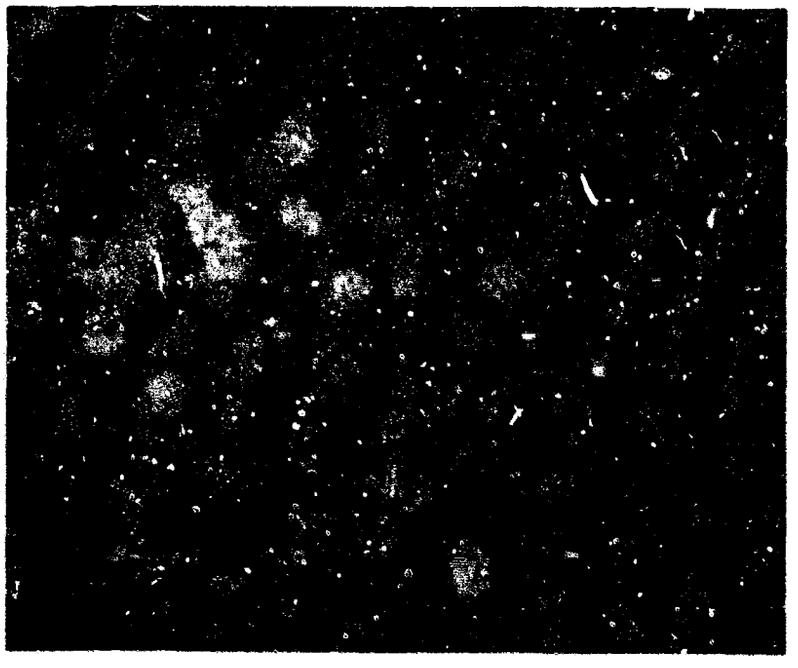

Figure 3. Illustration of Overlapping VEE Plaque.

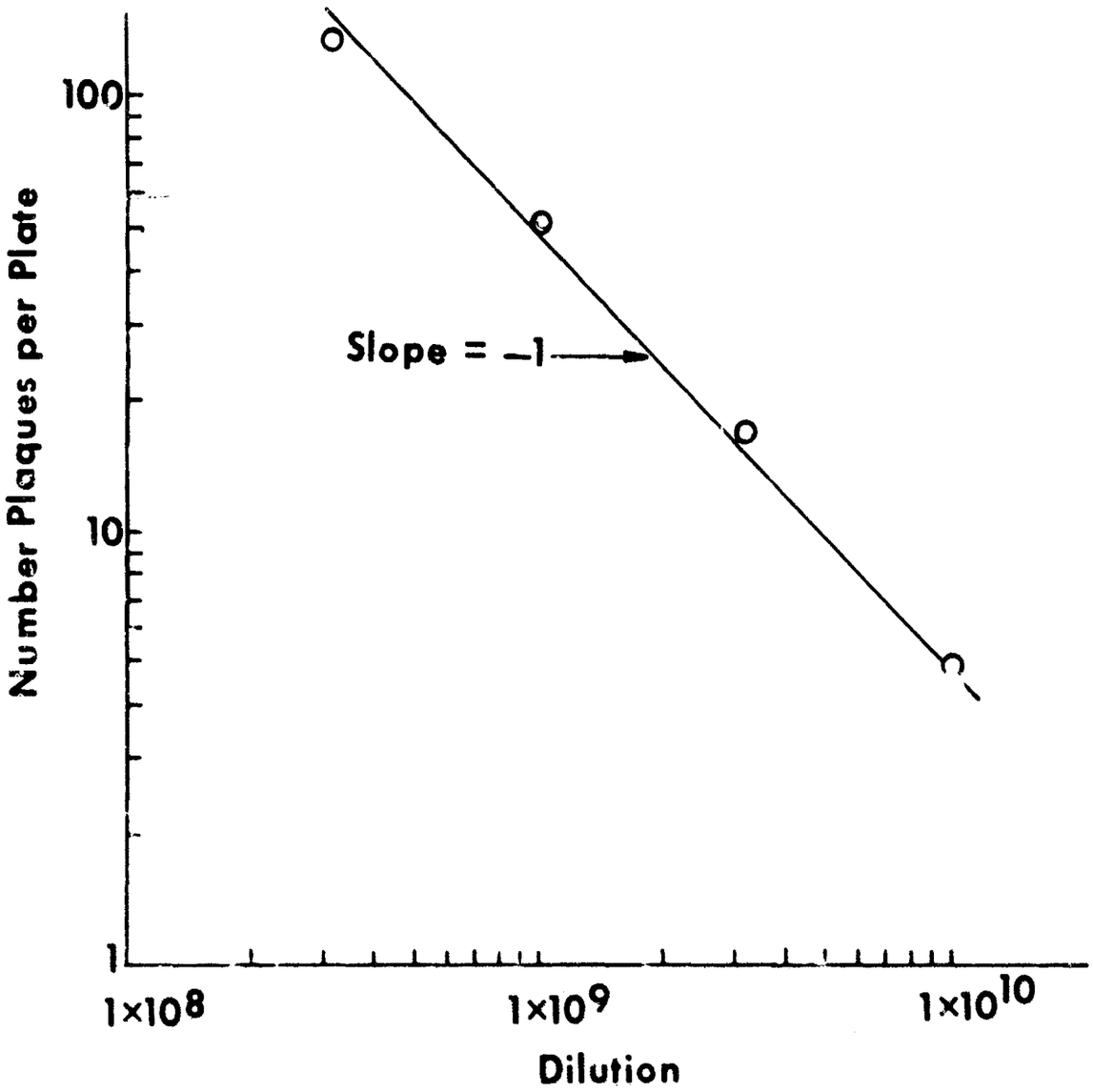

Figure 4. Relationship between Mean Number of Plaques per Plate and Dilution of Virus Plated.

The logarithm of the estimate of the PFU per gram was linearly related to the logarithm of the dilution plated. The plaque assays in terms of PFU per gram were transformed to their logarithms and are shown in Table 7. An analysis of these log PFU per gram, shown in Table 8, indicates no significant difference in estimates of PFU per gram among the dilutions. This means that the accuracy of the estimates of PFU per gram is independent of the dilution from which the estimate was made. The analysis of variance also indicates that statistically significant differences occurred only among the three products. There was no significant interaction between the product and the dilution. The variance of an estimated PFU per gram, based on five plates per dilution, was 0.0108.

TABLE 7. LOGARITHM OF ESTIMATE OF PFU PER GRAM

Rep	Dried Product	Log Dilution 8.5	9.0	9.5	10.0
I	A	11.2458	11.3364	11.4242	11.5797
	B	11.1875	11.1846	11.1627	10.9542
	C	11.4030	11.5693	11.5453	11.5440
II	A	11.4329	11.4927	11.6003	11.4771
	B	11.1665	11.0899	11.0314	11.2041
	C	11.4763	11.5843	11.5530	11.3222

TABLE 8. ANALYSIS OF VARIANCE OF LOG PFU PER GRAM

Line	Source	df	SS	MS	Error Line	F
1	Reps (R)	1	0.003601	0.003601	9	<1
2	Product (P)	2	0.669529	0.334764	9	30.98[a/]
3	Dilutions (D)	3	0.016741	0.005580	9	<1
4	P x D	6	0.074519	0.012429	9	1.15
5	R x P	2	0.020103	0.010051	7	<1
6	R x D	3	0.008313	0.002771	7	<1
7	R x P x D	6	0.090446	0.015074		
8	TOTAL	23	0.883312			
9	5 + 6 + 7 (Error)	11	0.118862	0.010806		

a. P <0.001

The mouse assay data (shown in Table 9) are summarized in the form of the six estimates of log $MICLD_{50}$ per gram in Table 10. The analysis of variance of the log $MICLD_{50}$ per gram values is shown in Table 11. The variance of these values, 0.0165, is comparable to 0.0108, the variance of the log PFU per gram from Table 8.

TABLE 9. RESPONSE RATIO OF MICE BY REPLICATE, PRODUCT, AND LOG DILUTION

Replicate	Product	Log Dilution 9.0	9.5	10.0	10.5
I	A	5/8[a]	5/8	2/8	1/8
	B	5/8	8/8	2/8	0/8
	C	8/8	7/8	5/8	0/8
II	A	5/8	4/8	3/8	0/8
	B	6/7	5/8	1/7	0/8
	C	8/8	6/8	5/8	3/8

a. No. dead/no. injected.

TABLE 10. ESTIMATES OF LOG $MICLD_{50}$ PER GRAM AND VARIANCES BY REPLICATE AND PRODUCT

Replicate	Product	Log $MICLD_{50}$/g	Variance
I	A	11.1736	0.12124
	B	11.0054	0.05730
	C	11.5057	0.01120
II	A	11.0762	0.01657
	B	10.9704	0.04699
	C	11.7388	0.04666
		Mean	0.04999

TABLE 11. ANALYSIS OF VARIANCE OF LOG $MICLD_{50}$ PER GRAM

Line	Source	df	SS	MS	Error Line	F
1	Reps (R)	1	0.00088	0.00088	3	<1
2	Product (P)	2	0.46035	0.23017	3	13.94
3	R x P (Error)	2	0.03302	0.01651		
4	TOTAL	5	0.49425			

The above data were not sufficient to establish a relationship between the numbers of PFU and $MICLD_{50}$ per unit (g or ml) of viral product, so a second investigation of four additional VEE products (D, E, F, and G) was conducted. Duplicate assays of the products were performed by both the plaque and mouse assay methods, using three plates and eight mice at each dilution.

A simple mathematical model, based on traditional microbiological concepts, leads to an expected slope of unity between log $MICLD_{50}$ and log PFU. Let Y_1 = log $MICLD_{50}$ and X = log concentration. For a dilution series, it is postulated that Y_1 is linearly related to X by $Y_1 = a_1 + b_1X$. Likewise for Y_2 = log PFU, it is postulated (and demonstrated here) that $Y_2 = a_2 + b_2X$. By simple substitution, Y_1 is linearly related to Y_2 according to $Y_1 = (a_1 - a_2/b_2) + (b_1/b_2)Y_2$ or $Y_1 = (a_1 - a_2) + Y_2$ if $b_1 = b_2 = 1$. This expectation of unit slope was achieved with this experiment.

The mean values for the duplicate assays by lot and type of VEE product are shown in Table 12. To verify that there is in fact a 1:1 relationship between plaque count and dilution in these data (i.e., that $b_2 = 1$), the linear regressions of log mean plate count on log dilution for 18 of the lots were computed and are presented in Table 13. No differences in the regression coefficients (b_2) among products were noted, and the number of b_2 differing significantly from 1.0 was about that expected by chance alone. The mean variance, s^2, about the regression line averaged over samples and products, was 0.0095 and is comparable to the variance of 0.0108 reported above.

TABLE 12. LOG PFU AND LOG $MICLD_{50}$ PER GRAM OR MILLILITER BY LOT AND PRODUCT[a]

Lot	D		E		F		G	
	Log PFU	Log $MICLD_{50}$	Log PFU	Log $MICLD_{50}$	Log PFU	Log $MICLD_{50}$	Log PFU	Log $MICLD_{50}$
	per ml		per ml		per g		per g	
1	9.64	9.70	9.68	9.91	10.68	10.63	-	-
2	10.15	9.98	10.19	10.03	10.88	11.00	11.15	10.91
3	10.17	9.74	9.85	9.90	10.64	10.66	10.55	10.38
4	10.01	9.85	10.18	10.26	10.76	10.53	10.78	10.69
5	9.86	9.76	9.85	9.86	11.03	11.10	11.12	11.20
6	10.24	9.89	10.18	10.25	11.06	11.07	10.91	10.86
7	10.11	9.98	10.11	10.10	10.95	10.85	10.85	11.02
8	9.72	10.09	9.84	10.18	10.90	10.48	10.87	10.57
9	9.93	10.22	9.87	9.53	10.80	10.70	11.04	10.53
10	10.25	9.95	10.23	10.05	11.33	11.12	11.33	10.98
11	10.39	9.82	10.34	9.72	10.98	10.94	11.07	10.84
12	10.42	9.99	10.46	10.12	10.75	10.56	11.11	11.11
13	10.11	9.88	9.91	9.72	10.72	10.83	11.26	10.66
14	10.37	9.36	10.42	10.12	-	-	-	-
15	10.41	10.50	10.44	10.49	11.44	11.18	11.56	11.06
16	10.03	9.46	10.17	9.46	11.29	10.82	10.92	10.60
17	9.94	9.87	9.98	9.99	10.64	10.81	11.20	11.12
18	10.02	10.04	10.11	10.01	11.31	10.88	11.18	10.97
19	10.21	9.75	10.29	10.03	-	-	-	-
20	10.33	10.01	10.54	9.65	11.23	10.90	11.38	11.01
21	10.21	9.91	10.14	10.17	11.45	11.01	11.27	10.95
22	10.21	9.57	10.26	9.61	11.26	10.59	11.17	10.31
23	10.36	9.97	10.35	10.78	11.39	11.33	11.35	11.03
24	10.32	10.49	10.40	10.35	11.42	11.24	-	-
25	10.45	10.33	10.41	10.31	-	-	-	-
26	10.32	9.78	10.17	9.53	10.54	10.87	11.01	10.66
27	10.43	10.13	10.55	10.03	11.34	11.44	11.34	11.22
28	10.55	10.37	10.55	10.54	11.38	10.98	11.43	10.89
29	10.55	9.98	10.43	9.76				
30	10.43	10.50	10.47	10.22				
31	10.43	10.01	-	-				
32	10.30	10.11	10.25	9.83				
33	10.38	10.06	10.54	9.85				
34	10.35	9.64	10.23	10.02				
35	10.41	10.19	10.38	10.26				
36	10.50	9.89	10.46	9.95				
37	9.95	10.26	10.06	10.06				
Mean	10.23	9.97	10.20	10.02	11.05	10.90	11.12	10.85

a. D and E = Liquid
F and G = Dried

TABLE 13. LINEAR REGRESSION COEFFICIENTS (b_2) OF LOG MEAN PLATE COUNTS (Y_2) ON LOG DILUTION (X) BY LOT AND TYPE OF PRODUCT

Lot	D	E	F	G
1	1.17	1.06	1.11	0.99
2	1.14	1.07	1.16	1.08
3	1.25[a]	1.01	0.76	1.07
4	1.05	0.85	0.96	1.01
5	0.82	0.88	0.98	0.98
6	0.83[a]	1.08	0.93	0.89
7	0.91	0.90	0.87[b]	1.06
8	1.19	0.93	0.75[a]	0.88
9	0.97	0.89	1.03	0.63[b]
10	0.94	0.81	0.94	0.96
11	0.93	1.12	1.10	1.11
12	1.03	1.06	1.07	1.00
13	1.24[a]	1.04	1.05	0.97
14	1.07	0.92	0.77[a]	1.23
15	0.83	1.12	0.99	1.05
16	1.16	1.12	0.89	1.08
17	1.26	1.45	1.00	1.18
18	1.08	0.92	1.02	0.94
Mean	1.05	1.01	0.97	1.01

Means for All Lots and Products

Statistic	Symbol	Mean	Largest	Smallest
Regression Coefficient	b_2	1.0078	1.25	0.63
Standard Error of b_2	$SE(b_2)$	0.0862	0.2037	0.0158
Variance about regression line	s^2	0.00953	0.04148	0.00024

a. Regression coefficients significantly different from 1.00 ($P \leq .05$).
b. Regression coefficients significantly different from 1.00 ($P \leq .01$).

To establish the relationship between log $MICLD_{50}$ and log PFU per g or ml of product, separate linear regressions were computed for each product. To simplify matters, it is convenient at this point to change the notation scheme. We now consider log PFU per g or ml as the independent variable (X_3) and log $MICLD_{50}$ per g or ml as the dependent variable (Y_3). Table 14 shows the estimates of the parameters of the new regression equation $Y_3 = a_3 + b_3X_3$ where a_3 is the Y intercept and b_3 the slope of this relationship. These b_3 are quite different from the expected unit slope, but are typical of the results obtained when a narrow range of concentration is studied. By combining all products, a range of approximately two logs was obtained that gave more usable results. The usual least squares estimate of the linear regression of Y_3 on X_3, which assumes that the independent variable is measured without error, is generally satisfactory even when X is known to be subject to experimental error, provided that the errors in X are small relative to the errors in Y. In data such as these, where the errors in X and Y are of approximately the same magnitude, the errors in X are sufficiently large to introduce bias into the estimate of the regression equation. Wald's analysis,[15] which corrects for the bias due to errors in X, gave the regression equation $Y_3 = -0.53 + 1.03\ X_3$, which is shown as a solid line in Figure 5. The regression coefficient b_3 of 1.03 is clearly not significantly different from the anticipated value of 1.00. The correlation coefficient between log PFU and log $MICLD_{50}$ of 0.8375 was of reasonable magnitude but not outstanding.

The Y intercept, i.e., the estimated value of Y when X equals zero, is often difficult to interpret biologically, particularly when the experimental values of X are a considerable distance from zero. Of greater importance are the relative values of X and Y over the range of values covered by this investigation. Between 9.6 and 11.6 log PFU per g or ml, the plaque assays were about 0.2 log higher than the mouse assays. This indicates that the plaque assay is slightly more sensitive than the mouse assay over the concentrations covered in this investigation. Assays of the dried product were less variable than the assays of the liquid materials, as shown in Figure 5.

TABLE 14. ESTIMATES OF THE PARAMETERS OF REGRESSIONS OF LOG $MICLD_{50}$ ON LOG PFU PER GRAM OR MILLILITER

Product	a_3	b_3
D	6.76	0.31
E	6.25	0.37
F	5.22	0.51
G	4.32	0.59

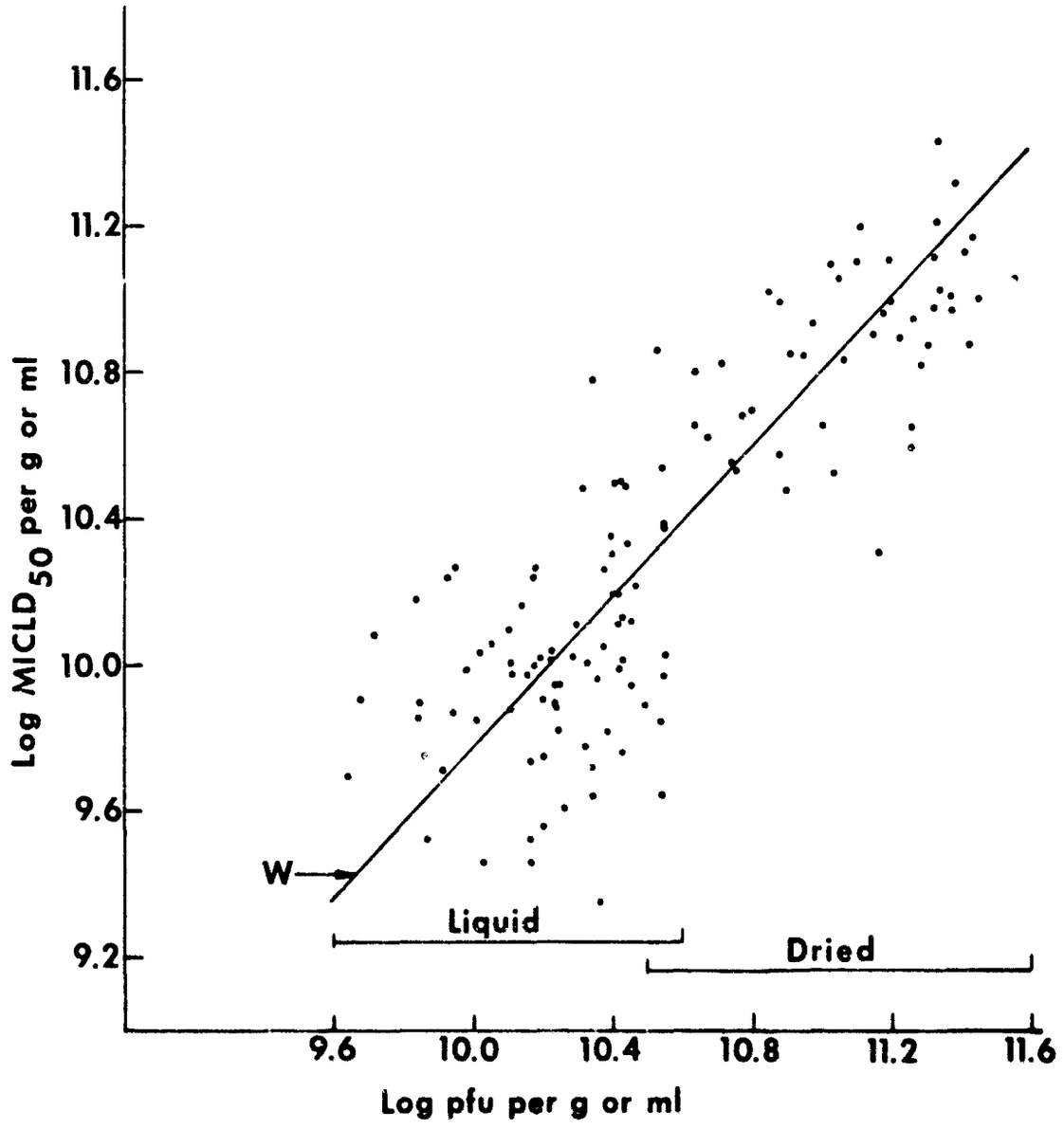

Figure 5. Relationship between Log $MICLD_{50}$ (Y_3) and Log PFU (X_3) per Gram or Milliliter after Correction for Errors in X_3.

IV. DISCUSSION

In this study the substitution of Tris buffer for the bicarbonate buffer in the tissue culture system was satisfactory for plaque formation and obviates the use of a CO_2 atmosphere. In initial experiments during this study with Tris buffer, the virus titers were approximately one log lower than those in mice. These assay results are approximately the same as those reported by Hardy and Hearn[1] with a bicarbonate buffer system and a CO_2-containing atmosphere.

In later experiments several environmental variables were studied in an effort to enhance plaque formation. The standard procedure was modified by incorporating the conditions that were found to be most favorable for plaque formation. The sensitivity of the plaque assay was enhanced approximately one log by modifying the standard procedure as follows: (i) serially diluting the virus in HIB at pH 6.0, (ii) adsorbing the virus at room temperature (25 C), and (iii) extending the adsorption time to 4 hours.

This modified plaque technique was used to determine the virus concentration of VEE products. Statistical analysis of the data shows that the plaque assay has the following desirable characteristics. (i) The distribution of variance of the plaque count differs only slightly from Poisson. Theoretically, data would show exact Poisson distribution; however, that would leave no room for other sources of error, e.g. dilution, technician, and sheet thickness. The existence of error slightly greater than Poisson indicates that these other sources of variance do in fact exist, but in this particular situation have very slight effect. (ii) Plaque counts reflect almost exactly the effects of dilution, i.e., the mean counts from plates of virus samples at the 7.5 dilution were uniformly one log lower than counts from plates at the 8.5 dilution. (iii) A comparison of the plaque-counting method with the mouse titration showed a linear relationship between log plaque units and log mouse units that further substantiates the hypothesized 1:1 ratio. The plaque assay was slightly more sensitive (0.20 log) than the mouse assay; this is in agreement with Dulbecco and others in that the plaque assay should be more sensitive because the monolayers of tissue culture are presumed to lack the natural and acquired defense mechanisms present in the mouse.

On the basis of experimental studies with poliomyelitis virus, Dulbecco and Vogt[16] ". . . defined the concept of the plaque-forming particle as a physical and biological unit. Since the plaque-forming titer of a virus particle is of the same order of magnitude as the infectious titer determined by other methods, the plaque-forming particle can be identified with the infectious particle. Furthermore, since the operational definition of a virus particle rests on its infectivity, this infectious particle is the virus particle." Therefore, the infectivity of VEE virus can be equally determined either in tissue culture (PFU) or in mice ($MICLD_{50}$).

This report shows that environmental factors influence the host-virus interactions of susceptibility and resistance at the cellular level. For example, pretreatment of the virus particle in HIB at pH 6.0 enhances the susceptibility of the chick fibroblast cells to infection.

This information might well apply in developing a procedure for isolating the virus from human suspects. If the virus is present at a low concentration in the sample obtained from the human suspect, one would expect a higher incidence of isolating the virus in tissue culture if the sample were first treated in HIB at pH 6.0. Another application of this information might be pretreating seed virus (VEE) used to infect cells when growing the virus in a tissue culture system. By increasing the susceptibility of the cells to infection, the log phase may be shortened and less time may be required to obtain maximum virus concentration.

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13 ABSTRACT

A reproducible plaque assay procedure for the quantitation of Venezuelan equine encephalomyelitis (VEE) virus in chick fibroblast monolayer cultures is described that is slightly more sensitive (0.20 log) than the mouse assay. The standard tissue monolayer assay was modified by using tris(hydroxylmethyl)-aminomethane (Tris) as a buffer for the agar overlay. Chick fibroblast monolayer cells infected with VEE virus can be maintained under the Tris agar overlay for the time necessary for plaque formation, using a conventional laboratory incubator without a carbon dioxide atmosphere. The adsorption of the virus to chick fibroblast cells was more efficient when the virus inoculum was serially diluted in heart infusion broth at pH 6.0 and when the virus adsorbed for 4 hours at 25 C before the agar overlay was added. Statistically, the plaque and mouse assays showed a linear relationship, 1:1 ratio, between log plaque-forming units and log mouse intracerebral LD_{50} units; thus, the plaque assay can replace the mouse assay for determining virus concentration of VEE products.

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